PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION International Bureau **B5**

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 : C07K 15/28, G01N 33/563, H61K 39/395, C12P 21/08 A1

(11) International Publication Number: WO 91/034

(43) International Publication Date: 21 March 1991 (21.03.

(21) International Application Number: PCT/GB90/01335

(22) International Filing Date: 29 August 1990 (29.08.90)

(30) Priority data:

8919485.6	29 August 1989 (29.08.89)	GB
9001949.8	29 January 1990 (29.01.90)	GB

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF SOUTHAMPTON [GB/GB]; Highfield, Southampton SO9 5NH (GB).

(72) Inventor; and

(75) Inventor/Applicant *(for US only)* : GLENNIE, Martin, John [GB/GB]; The Tenovus Research Laboratory, Southampton General Hospital, Tremona Road, Southampton SO9 4XY (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp Co, 14 South Square, Grays Inn, London WC1R 5I (GB).

(81) Designated States: AT, AT (European patent), AU, BB, I (European patent), BF (OAPI patent), BG, BJ (OA patent), BR, CA, CF (OAPI patent), CG (OAPI paten CH, CH (European patent), CM (OAPI patent), DE DE (European patent)*, DK, DK (European paten ES, ES (European patent), FI, FR (European paten GA (OAPI patent), GB, GB (European patent), HU, I (European patent), JP, KP, KR, LK, LU, LU (Europe patent), MC, MG, ML (OAPI patent), MR (OAPI p tent), MW, NL, NL (European patent), NO, RO, SI SE, SE (European patent), SN (OAPI patent), SU, T (OAPI patent), TG (OAPI patent), US.

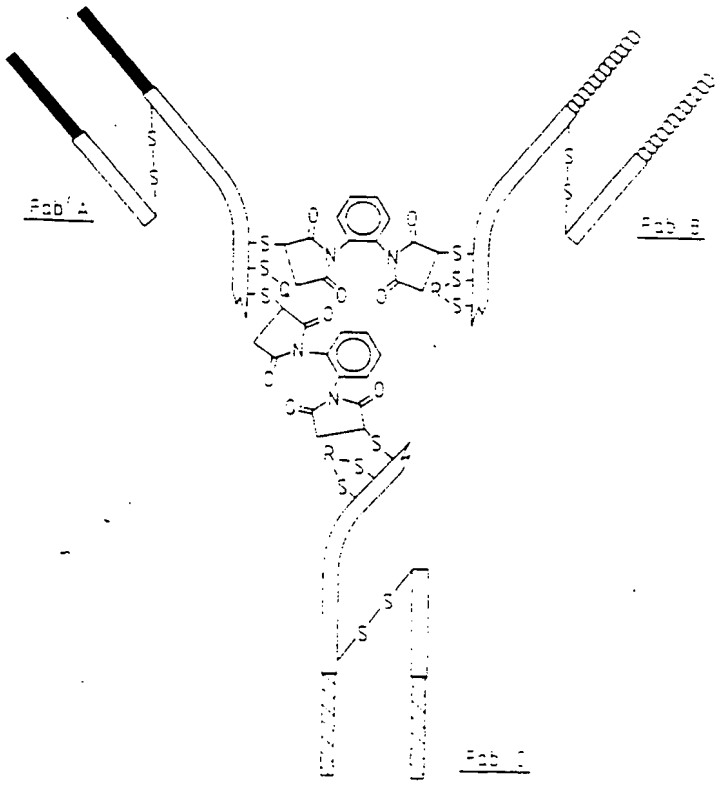
Published

With international search report.

(54) Title: BI-OR TRISPECIFIC $(FAB)_3$ OR $(FAB)_4$ CONJUGATES

(57) Abstract

Novel trimeric and tetrameric antibodies are disclosed, including bispecific and trispecific $F(ab)_3$ and $F(ab)_4$ antibodies. A simple and efficient method is described for the production of pure $F(ab')_3$ antibodies, in which the individual antibody Fab' fragments are joined via stable thioether linkages. Hybrid molecules were constructed from mouse monoclonal antibodies with specificities for targetting cytotoxic effectors (human peripheral blood T cells) against ^{51}Cr-labelled chicken red blood cells. Fab' fragments from two of the chosen antibodies were first coupled via their hinge-region SH groups using o-phenylenedimaleimide (oPDM), this bispecific fragment was then linked, again via the hinge region using oPDM, to a third Fab' fragment.



TRISPECIFIC F(ab')3 ABC

* See back of page

Bi-or trispecific $(Fab)_3$ or $(Fab)_4$ conjugates

Antibodies which are bispecific with respect to the antigen they recognize have been used successfully in a number of applications. In immunochemistry they have been used to cross-link cellular antigen and detecting agent such as ferritin or horseradish peroxidase, doing away with the antibody conjugates used in more conventional methods. Similarly they have been used as heterobifunctional protein cross-linkers for the immobilization of enzymes in a number of assays. Perhaps their greatest potential lies in their therapeutic use for the targeting of unwanted cells or pathogens by cytotoxic effector cells or pharmacologic agents such as drugs or toxins.

It has been demonstrated that bispecific $F(ab'\delta)_2$ antibodies, in which one Fab'δ arm is directed at a lymphoma cell and the other binds to a ribosome-inactivating protein, such as ricin A chain or saporin, can target a toxic agent to tumour cells both in vitro and in vivo and prevent further growth. For targeting effector T cells and polymorphonuclear leukocytes, bispecific antibodies have usually have been employed which cross-link the T cell receptor complex or the Fc receptor, respectively, onto the target cell and thereby mediate high levels of specific lysis. By using the appropriate derivatives in this way, it has been possible to show that both normal circulating T cells and single clones of cytotoxic T lymphocytes can be "armed" to destroy almost any specified target cell, and that lysis is independent of the major histocompatibility complex status of the cells involved. Furthermore, the bispecific antibodies do not simply serve to "glue" the two cell populations together, but in linking the effector and target cell actually trigger the lytic process.

In addition to therapeutic uses, bispecific antibodies have also been useful as tools for understanding some of the molecular

including genetically engineered Fv fragments, which may have engineered on multiple residues suitable for forming links between fragments. Such fragments will form Fv_3 and Fv_4 antibodies.

Antibodies of the invention may have specificities for any antigens against which antibodies can be raised or engineered. They find particular application in therapy, especially against tumour cells, but also have applications in assay techniques.

Preferably, however, at least one arm of the antibody specific for a marker on a target, which may be a target cell such as tumour cell, and at least one arm is specific for a marker on an effector, which may be an effector cell such as a T cell, lymphocyte or macrophage, or it may be another cell toxin such as a ribosome-inactivating protein, for example saporin, ricin A chain or intact ricin, or another therapeutic agent to which antibodies can be raised or engineered, such as daunomycin or adriamycin.

In the case of a trispecific antibody, the trispecificity allows it to at once bind to an effector cell and to activate it. The third arm binds to the target cell. It is preferred that two arms of the trispecific antibody are specific to T cells, one of the CD3 molecule and the other to an accessory surface molecule such as the CD2, CD4 or CD8. Alternatively, both arms may be specific for CD2. In that event, the two arms are specific for different epitopes on CD2 such as $T11_2$ and $T11_3$ or $T11_2$ and $T11_3$.

Also in accordance with the first aspect of the invention there is provided a process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating a first $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

any minor products which may have formed by oxidation or disulphide exchange, before fractionating an Ultrogel AcA44. In the preparation of trispecific antibody Fab_{SH} fragments are prepared from three antibodies to give $FabA_{SH}$ $FabB_{SH}$ and $FabC_{SH}$ fragments. The linkage of the Fab_{SH} fragments is effected by treating $FabA_{SH}$ fragments with oPDM to give $FabA_{mal}$ fragments. These are combined with untreated $FabB_{SH}$ fragments under cross-linking conditions to give bispecific $F(ab)_2AB$, as shown in Fig. 1. $FabC_{SH}$ fragments are similarly treated with oPDM, to yield $FabC_{mal}$ fragments, which are combined with the $F(ab)_2AB$ by means of an -SH group on the $F(ab)_2AB$ to give trispecific $F(ab)_3ABC$ antibody (see Fig. 2).

According to a second aspect of the invention there is provided a conjugate comprising an antibody according to the first aspect of the invention and an effector for which at least one of the arms of the antibody is specific. The invention also contemplates a process for the preparation of such a conjugate, in which the antibody is mixed with the effector.

The invention further contemplates a pack comprising an antibody according to the first aspect of the invention and, separately, an effector for which at least one of the arms of the antibody is specific.

The invention will be further described with reference to the example and to the figures, in which:

Figure 1 shows the postulated reaction between two Fab fragments to produce a bispecific $F(ab)_2$ antibody;

Figure 2 shows the proposed structure of a trispecific antibody according to the invention;

Figure 3 shows typical chromatography profiles obtained during the preparation of bispecific $F(ab'\gamma)_3$ and trispecific $F(ab'\gamma)_3$;

in supplemented RPMI [RPMI 1640 Medium containing the same supplements as the DMEM, but with the FCS replaced by 10% normal human serum which had been incubated at 56°C for 30 min to destroy any complement-mediated cytotoxic activity].

Antibodies:

A mouse IgG1 monoclonal antibody, $E_{11}C_{12}$, reacting with chicken red blood cells (CRBC) was raised using conventional hybridoma technology. BALB/c mice were immunized in a protocol which delivered CRBC (approx 10^9) s.c. in CFA and IFA (Difco, Detroit, MI) on days 0 and 14 respectively, and i.p. in DMEM on day 24. Four days later splenic mononuclear cells were fused with the NS-1 (P3/NS-1/1-Ag4.1) mouse myeloma line at a ratio of 2:1 by using a standard somatic fusion protocol with polyethylene glycol 4000 (E.Merck, Darmstadt, Germany). Hybridoma cells secreting anti-CRBC antibody were identified by immunofluorescence staining and flow cytometry as described previously and cloned by limiting dilution.

Additional hybridoma cell lines producing the antibodies OKT1 (CD5), OKT3 (CD3) and OKT11 (CD2) were obtained from the American Type Culture Collection (ATCC, Rockville, Maryland), and the hybridoma 3G8 (CD16) was a gift from Dr. D. Segal, NIH, Bethesda, Maryland.

All hybridoma cells were expanded as ascitic tumors in pristane-primed (BALB/c x CBA) F1 mice. The 7S IgG fraction of monoclonal ascites was isolated as described by precipitation in 2 M ammonium sulfate, followed by ion exchange chromatography on Trisacryl-M-DEAE (LKB-Produkter AB, Bromma, Sweden).

Antibody $F(ab'\delta)_2$ fragments from IgG were prepared by limited proteolysis with pepsin at pH 4.1-4.2 in 0.1 M sodium acetate. The reaction being monitored at regular intervals by rapid fractionation of 100 µg samples on a GF 250 HPLC column (Zorbac), and then, when less than 10% of the IgG remained, the digestion was stopped by adjusting the pH to 8.0 with saturated Tris-base and the products fractionated on Ultrogel AcA44 (LKB).

reaction conditions for linking the Fab'γ_{mal} to the F(ab'γ)$_{2SH}$ were similar to those used Example 1: bispecific F(ab'γ)$_2$ and Fab'γ_2 fragments from the third antibody at 5 mg/ml in 0.2 M Tris-HCl buffer, pH 8.0, containing 10 mM EDTA were reduced by addition of 20 mM 2-mercapoethanol for 30 min at 30°C. The samples were chilled to 4°C and run through Sephadex G-25 which had been equilibrated in a buffer of 50 mM sodium acetate, pH 5.3, containing 0.5 mM EDTA. The third Fab'γ_{SH} antibody species was then maleimidated using o-PDM as in the bispecific antibody preparation and finally the bispecific F(ab'γ)$_{2SH}$ and Fab'γ_{mal} were mixed together at a weight ratio of 1:4 for 18 hours at 4°C. Following chormatography on AcA44, 150 kDa-sized material (i.e. F(ab'γ)$_3$) was harvested and concentrated.

Trispecific F(ab'γ)$_4$ was also generated during the trispecific F(ab'γ)$_3$ preparation. It emerged from the AcA44 column at a position which corresponded to that of a protein with a molecular weight of approximately 200 kDa. This size is consistent with the joining of four Fab'γ fragments during the reaction. Apparently, the bispecific F(ab'γ)$_2$ has conjugated with two Fab'γ_{mal} fragments from the third antibody.

The final products in the reaction mixtures were reduced and alkylated with 20 mM 2-mercaptoethanol and 25 mM iodoacetamide respectively to remove any minor products which may have formed by oxidation or disulphide exchange, before fractionating on Ultrogel AcA44.

For targeting cytotoxic agents to unwanted cells:

toxin ** x Ig x CD19

toxin x Ig x CD22

toxin x Ig x CD37

** toxin = ribosome inactivating proteins such as saporin or ricin

The proposed structure of a trispecific $F(ab'\gamma)_3$ antibody of the invention specific for antigens A, B and C is shown in Fig. 2. Although not shown, some of the γ-L chain disulfide bonds will be reduced during the preparation. Such reduction is known not to compromise antigen binding activity in Fab fragments. Before the final product was alkylated with iodoacetamide, one hinge-region sulphydryl (-SH) group remained, offering the potential for linking at least one more $Fab'\gamma_{mal}$ fragment yielding $F(ab'\gamma)_4$, as mentioned above. The groups joined to the cysteinyl sulfur are: Q is carboxyamidomethyl 2, a blocking group; and R is o-phenylenedisuccimimidyl.

Typical chromatography profiles obtained during the preparation of (a) bispecific $F(ab'\gamma)_3$ and (b) trispecific $F(ab'\gamma)_3$ derivatives are shown in Fig. 3 (a) and (b) respectively

(a) A reaction mixture containing Fab'_{mal} and Fab'_{SH} at a weight ratio of 2:1 was reduced and alkylated and then fractionated on Ultrogel AcA44 in 0.2 M Tris HCl, pH 8.0. The unreacted Fab' fragments and the bispecific $F(ab'\gamma)_2$ and $F(ab'\gamma)_3$ are indicated.

(b) A reaction mixture containing bispecific $F(ab'\gamma)_{2\ SH}$ and Fab'_{mal} at a weight ratio of 1:4 was reduced and alkylated and then fractionated as for (a). $Fab'\gamma$, $F(ab'\gamma)_2$, $F(ab'\gamma)_3$ and putative $F(ab'\gamma)_4$ are indicated.

(a) shows lytic activity of the CD3 x CRBC antibody (100 ng/ml) in 4 and 8 hour assays using fresh PBL from six healthy donors.

(b) shows lysis of CRBC using PBL from one healthy donor and various concentrations (as indicated) of CD3 x CRBC and CD16 x CRBC in 4, 8 or 21 hour assays. The assay time is indicated on each of the CD3 x CRBC titration curves, but is omitted from those of the CD16 x CRBC derivative due to their proximity.

Note that while there is considerable variation between donors, all have shown appreciably higher levels of lysis in the longer assay. With two of the donors it was only in this longer 8 hour assay that significant release of ^{51}Cr could be measured at all. This result is confirmed and extended for one donor in Figure 4b, which shows that near maximal lytic activity was approached in eight hours with an antibody concentration of 100 ng/ml.

A graph showing redirected antibody dependent cellular cytotoxicity of ^{51}Cr-labeled CRBC by PBL and one or more bispecific $F(ab'\gamma)_2$ antibodies is shown in Fig. 5. Lysis was measured in an 8 hour assay using fresh PBL from one donor and the derivatives indicated.

Results indicated that while the CD3 x CRBC $F(ab'\gamma)_2$ lysed CRBC at concentrations as low as 4 ng/ml, almost 1000 times more CD2 x CRBC $F(ab')_2$ was necessary to achieve appreciable levels of cytotoxicity. Mixing the CD2 bispecific reagents with the CD3 x CRBC antibody resulted in no additive effects, giving similar levels of killing to those seen with the CD3 derivative alone.

Fig. 6 shows that the redirected cellular cytotoxicity against ^{51}Cr-labelled CRBC is considerably higher for bispecific $F(ab'\gamma)_3$ derivatives than for bispecific $F(ab'\gamma)_2$ derivatives. For example, the CD3 x CD3 x CRBC trimer was found to be up to 125 times more potent than the equivalent dimer, CD3 x CRBC, giving significant activity at concentrations below 0.1ng/ml.

cost of treatment and directly to the patient who will take a lower dosage of antibody.

To confirm that both the arms of the trispecific antibody specific for the effector were required for full activity we performed RCC assays in the presence of blocking Fab' δ antibody. Figure 9(a), shows the cytotoxicity of the CD2 x CD3 x CRBC antibody in the presence of, respectively, CD2 antibody, CD3 antibody and a mixture of CD2 antibody and CD3 antibody as blocker. The graph shows that when using CD2 antibody CD3 antibody at 500 μg/ml, a concentration known to be sufficient to block bispecific derivatives (Fig. 7) no reduction in the activity of this trispecific reagent was observed. It was only when both these blocking antibodies were included in the assay that any reduction in activity occurred. With Fab' from both CD2 antibody and CD3 antibody each 500 μg/ml, specific ^{51}Cr release was reduced from 65% to 15%. In a similar experiment the results of which appear in Fig. 9(b), the trispecific F(ab'γ)$_3$ antibody CD3 x CD5 x CRBC also demonstrated redirected cellular cytotoxicity which was much more resistant to blocking than a bispecific F(ab'γ)$_2$ (See Fig. 7). In this example however, the blocking was slightly more effective than that with the CD2 x CD3 x CRBC reagent. While this increased sensitivity to blocking could reflect a reduced avidity by the CD3 x CD5 x CRBC antibody, it is probably also affected by the relatively poor cytotoxic potency of this reagent.

Incorporation of [^{3}H]thymidine:

Proliferation of normal T cells in response to mitogenic antibody derivatives was assessed in vitro. Peripheral blood lymphocytes (PBL) isolated from Ficoll-Hypaque were cultured at 37^{o}C in 96-well, U-bottomed, microculture plates (10^5/well) in supplemented RPMI containing the various antibody derivatives, together with or without CRBC (200 μl /well). After 48 hours each well was pulsed for 16 hours at 37^{o}C with 1 μCi [^{3}H]thymidine (Amersham) and the incorporated radioactivity harvested onto glass microfibre filters and assessed as described previously. All experimental points were determined in triplicate.

required at least 20 times more free Fab'δ from the appropriate antibody to inhibit cytotoxicity than did equivalent $F(ab'\delta)_2$ reagent.

In the case of trispecific antibodies, it may be advantageous to have two antibody Fab arms reacting with the target cell and one Fab arm recruiting either a cellular effector or a pharmacological agent such as a toxin. As with the effector T cell, other cells, including B cells and monocytes, can be activated when bound by two antibodies reacting with the appropriate surface molecules. Activiated cells which show a high proliferative rate are also often more susceptible to destruction by pharmacological agents such as cytotoxic drugs and toxins; the more rapidly growing tumours, such as childhood acute lymphoblastic leukaemia, are often the most sensitive to conventional chemotherapy. Thus, a trispecific antibody, with two Fab arms directed at the target cell and one at a pharmacological agent, could first activate the target cell and then deliver a poison while it remained in a hypersensitive state.

In addition to the advantages of being able to activate effector or target cells, trispecific antibodies, because they have two Fab arms binding to one surface, also display an increased avidity for that surface. A trispecific $F(ab)_3$ derivative will cross-link two cell surfaces together significantly more strongly than a mixture of two bispecific $F(ab)_2$ antibodies. This advantage may also be applied to immunoassays including enzyme-linked immunosorbent assays or radio-immunoassays. In this situation a trispecific antibody with two different binding sites for a single antigen, such as an enzyme, protein or peptide, and a third Fab arm for a second protein, can be used as a single step cross-linker increased avidity.

Other immunoassays in which a trispecific $F(ab)_3$ derivatives may be useful include situations where it is necessary to capture three different immunogenic antigens, such as enzymes, proteins or peptides, into a tight immune complex. An enzyme and its substrate could be captured directly from solution onto a solid surface in this way.

Claims

1. A trimeric or tetrameric antibody.

2. A bispecific or trispecific antibody according to claim 1.

3. An antibody according to any preceding claim comprising at least one arm specific for a marker on a first moiety and at least one arm specific for a marker on a second moiety.

4. A trispecific antibody according to any preceding claim comprising one arm specific for a marker on a first moiety and two arms specific, respectively, for different markers on a second moiety.

5. An antibody according to claim 3 or 4 in which the first and second moieties are, respectively, a target cell and an effector.

6. An antibody according to claims 3 or 4 in which the first and second moieties are, respectively, an effector and a target cell.

7. A antibody according to claim 5 or 6 in which the effector is an effector cell.

8. An antibody according to claims 5 or 6 in which the effector is a therapeutic agent effective to destroy the target cell.

9. An antibody according to claim 8, in which the therapeutic agent is a conventional chemotherapeutic compound to which antibodies can be raised, such as daunomycin or adriamycin.

10. An antibody according to any of claims 5 to 9 in which the target cell is a tumour cell.

21. An $F(ab'\gamma)_3$ or $F(ab'\gamma)_4$ antibody according to any preceding claim.

22. A Fv_3 or Fv_4 antibody according to any of claims 1 to 17.

23. A process for the preparation of a bispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificty into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms; and

(iii) linking the first Fab arm from step (i) to two component Fab arms from step (ii) to give bispecific $F(ab)_3$.

24. A process for the preparation of a trispecific $F(ab)_3$ antibody comprising:

(i) dissociating an $F(ab)_2$ antibody fragment having a first specificity into its two component Fab arms;

(ii) dissociating a second $F(ab)_2$ antibody fragment having a second specificity into its two component Fab arms;

(iii) linking the first Fab arm from step (i) to the Fab arm from step (ii) to construct a bispecific $F(ab)_2$ antibody;

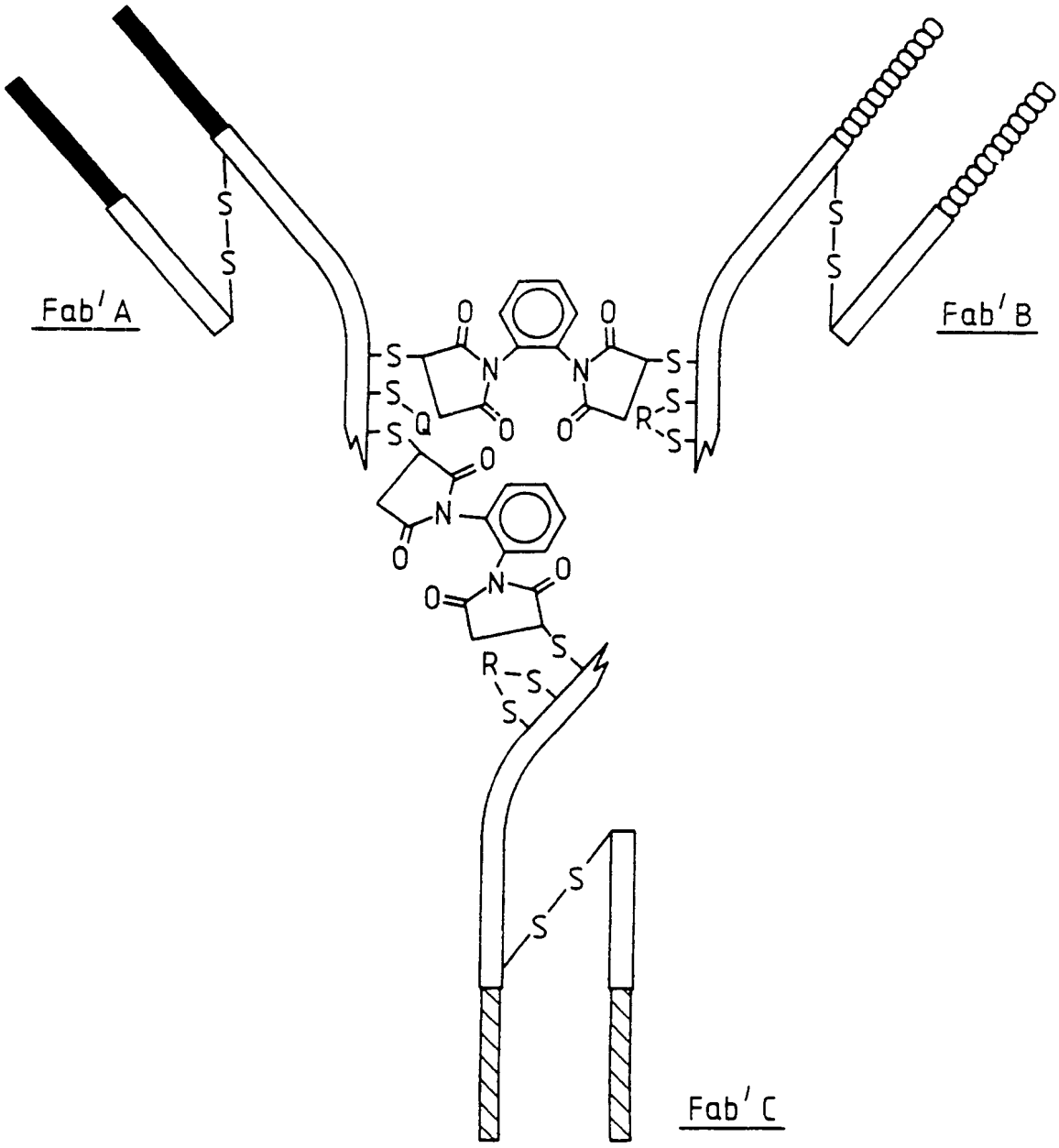
(iv) dissociating a third $F(ab)_2$ antibody fragment having a third specificity into its two component Fab arms; and

(v) linking the bispecific $F(ab)_2$ antibody from step (iii) to the Fab arm from step (iv) to give trispecific $F(ab)_3$.

according to any of claims 23 to 28; or a conjugate according to claim 29, or a conjugate produced by a process according to claim 30.

32. A pack comprising: a bispecific or trispecific antibody according to any of claim 1 to 22 or produced by a process according to any one of claims 23 to 28 and, separately therefrom, an effector for which at least one arm of the antibody is specific.

Fig. 2.



TRISPECIFIC $F(ab')_3$ ABC

Fig. 4.

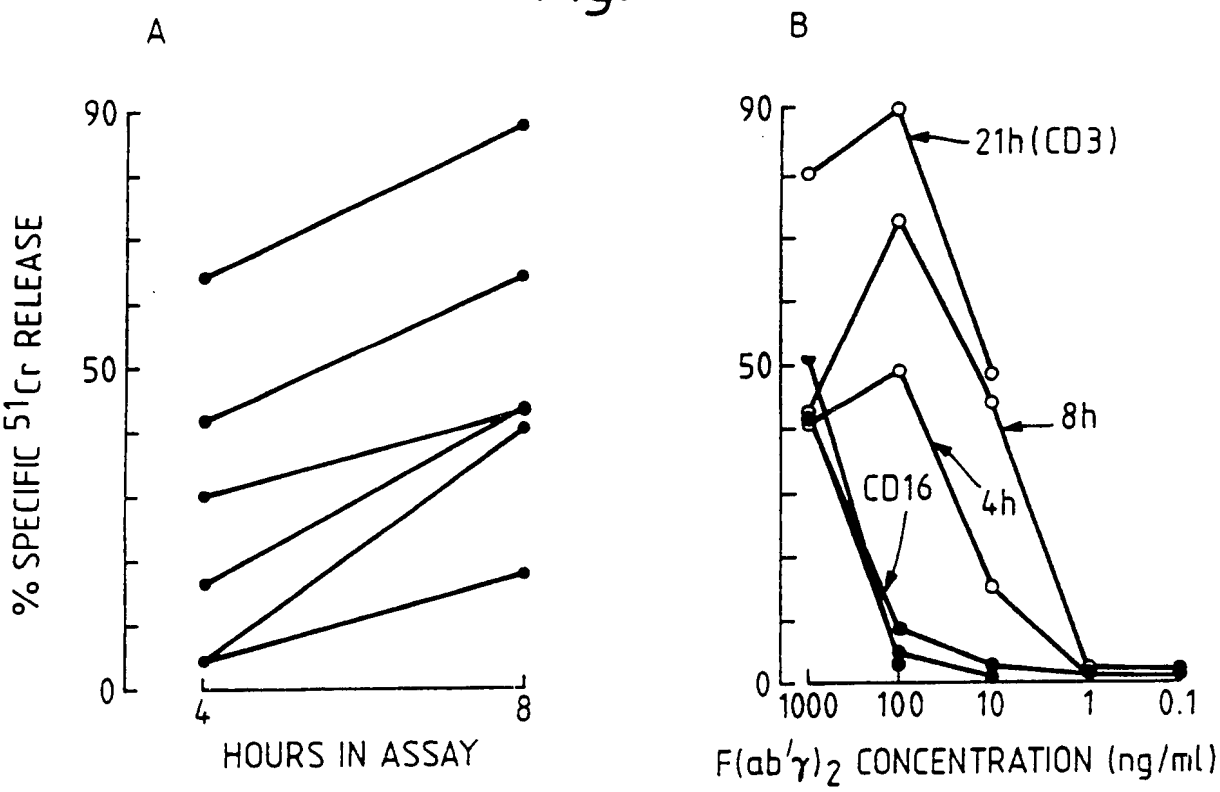

A
90
50
0
% SPECIFIC ^{51}Cr RELEASE
4
8
HOURS IN ASSAY
B
90
50
0
21h (CD3)
8h
4h
CD16
1000
100
10
1
0.1
F(ab'γ)2 CONCENTRATION (ng/ml)

Fig. 6.

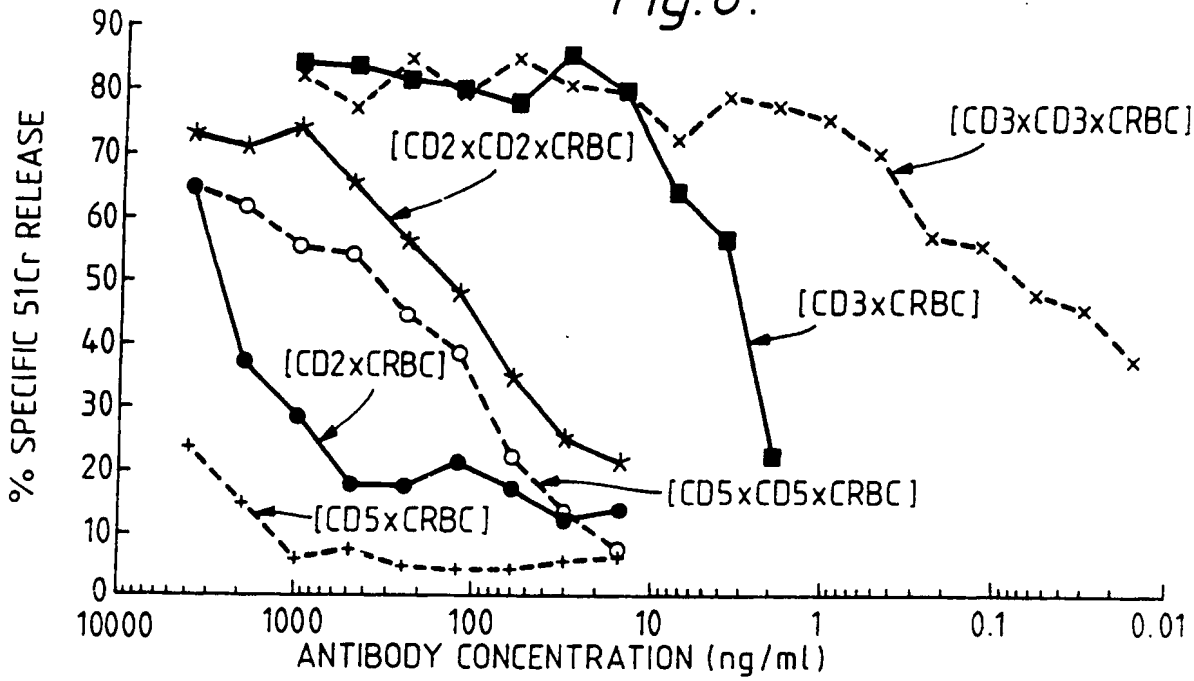

% SPECIFIC 51Cr RELEASE
90
80
70
60
50
40
30
20
10
0
[CD2xCD2xCRBC]
[CD3xCD3xCRBC]
[CD3xCRBC]
[CD2xCRBC]
[CD5xCD5xCRBC]
[CD5xCRBC]
10000
1000
100
10
1
0.1
0.01
ANTIBODY CONCENTRATION (ng/ml)

Fig. 7.

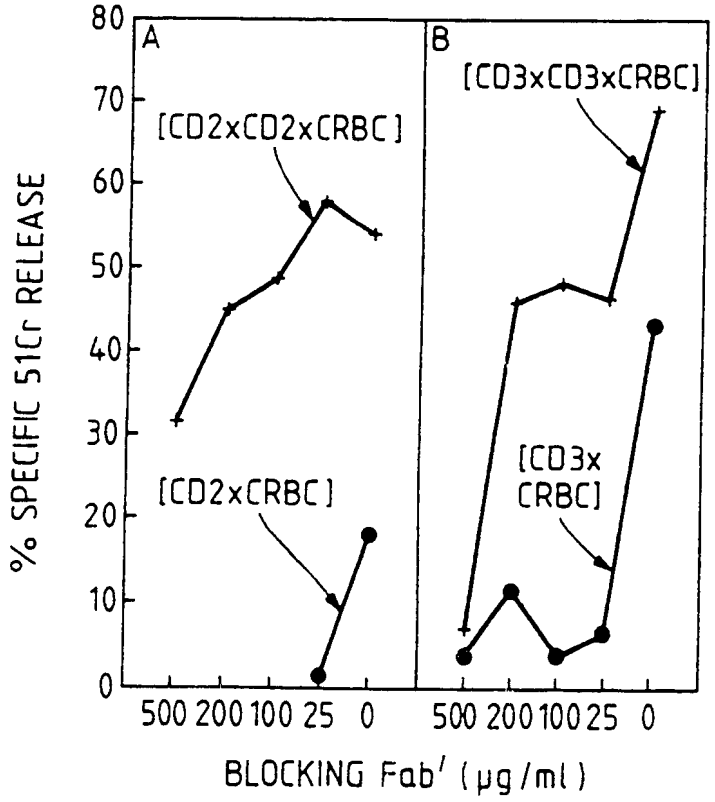
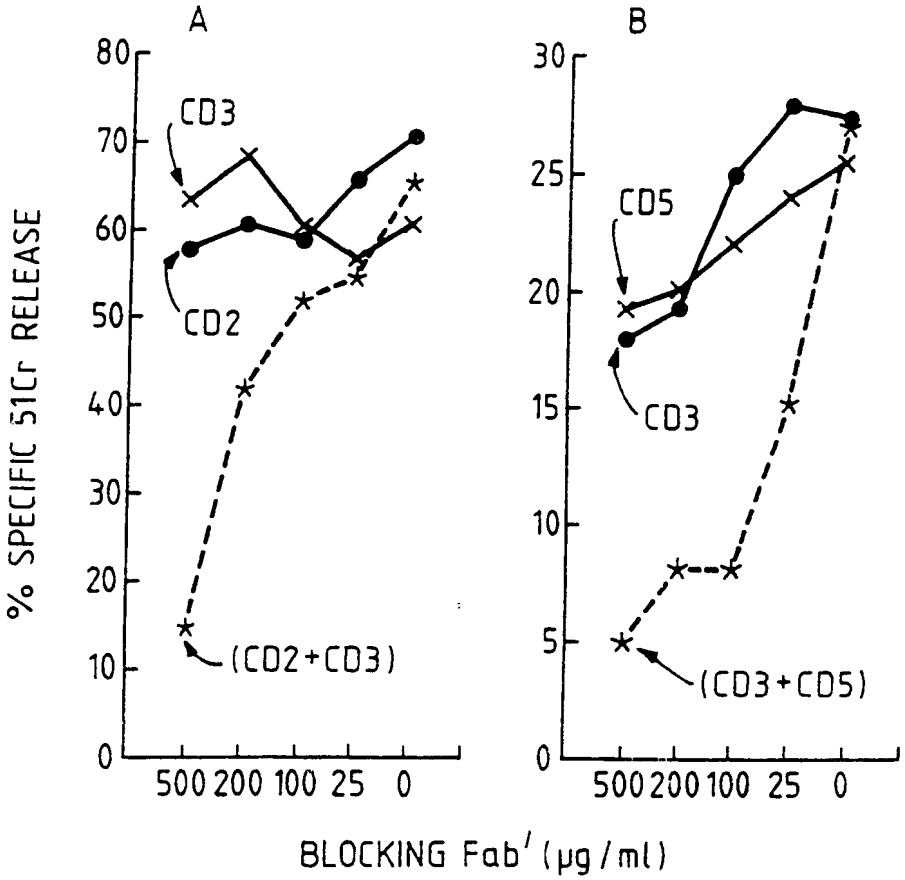

% SPECIFIC 51Cr RELEASE
80
70
60
50
40
30
20
10
0
A
B
[CD2xCD2xCRBC]
[CD3xCD3xCRBC]
[CD2xCRBC]
[CD3x
CRBC]
500 200 100 25 0
500 200 100 25 0
BLOCKING Fab' (µg/ml)

Fig. 9.


A
B
% SPECIFIC 51Cr RELEASE
CD3
CD2
(CD2+CD3)
CD5
CD3
(CD3+CD5)
500 200 100 25 0
BLOCKING Fab' (µg/ml)

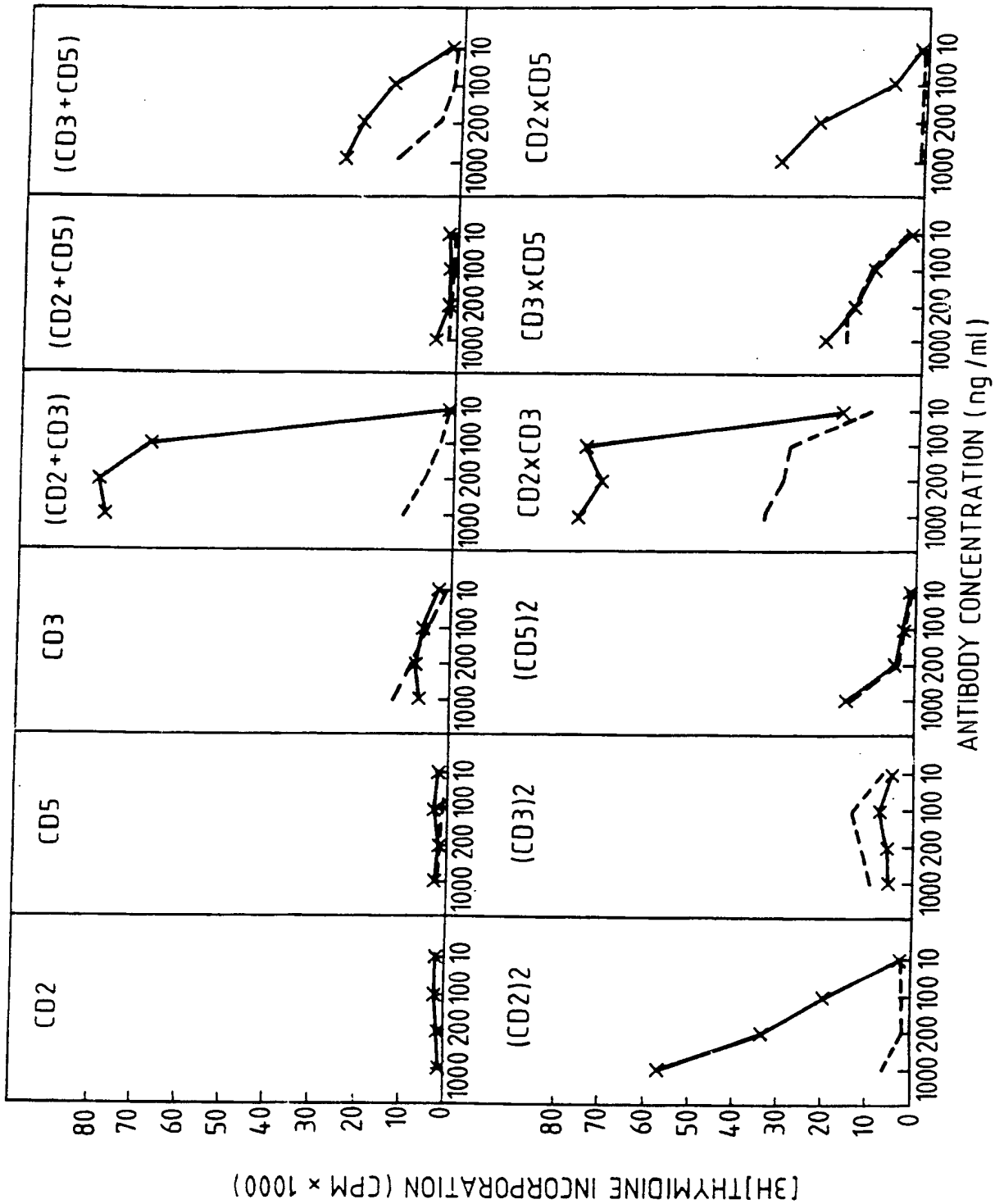
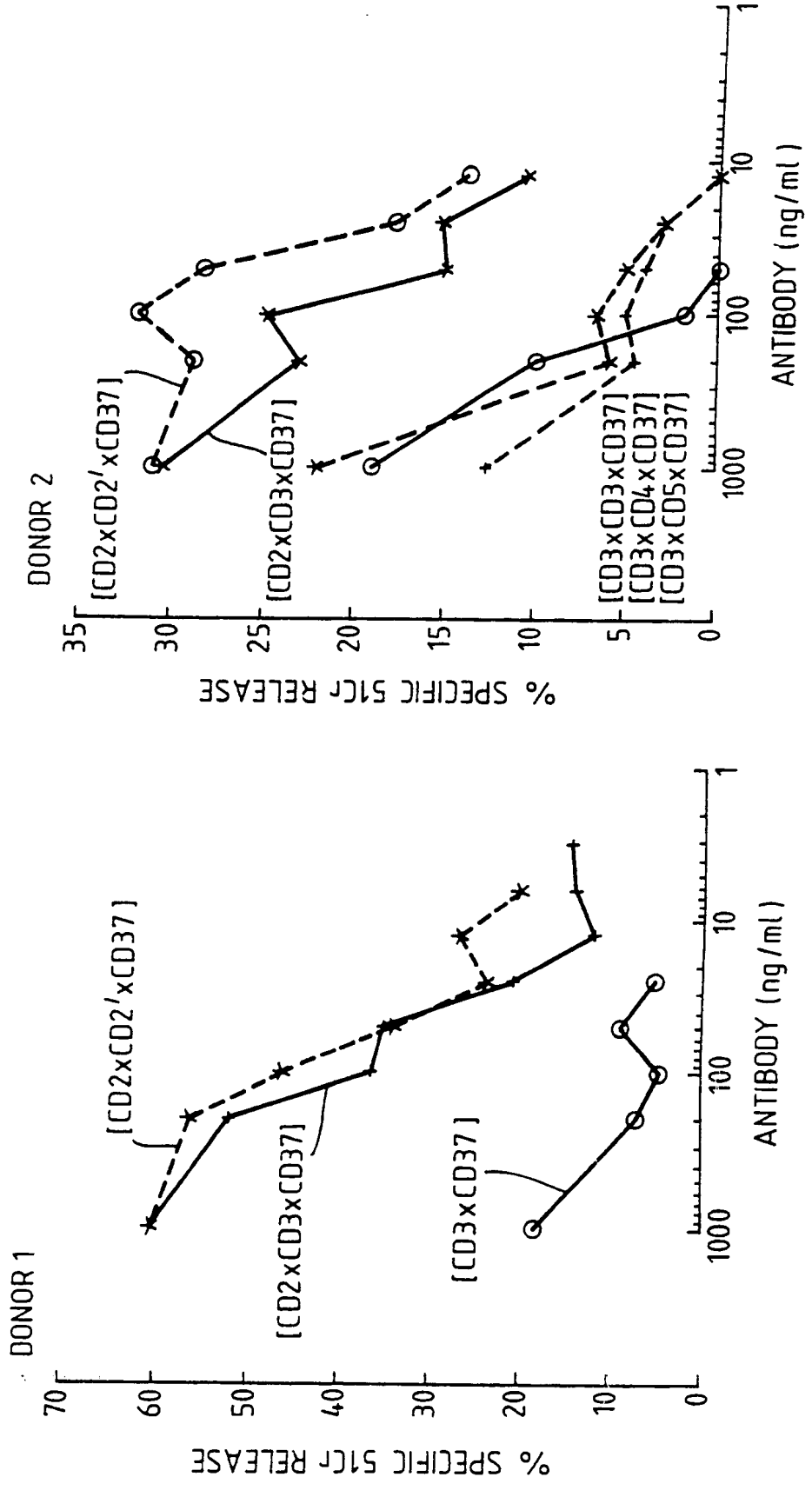

CD2
CD5
CD3
(CD2+CD3)
(CD2+CD5)
(CD3+CD5)
(CD2)2
(CD3)2
(CD5)2
CD2xCD3
CD3xCD5
CD2xCD5
0 10 20 30 40 50 60 70 80
1000 200 100 10
[3H]THYMIDINE INCORPORATION (CPM x 1000)
ANTIBODY CONCENTRATION (ng/ml)

Fig. 11.

Fig. 13.


DONOR 1
% SPECIFIC 51Cr RELEASE
70
60
50
40
30
20
10
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD37]
1000
100
10
1
ANTIBODY (ng/ml)
DONOR 2
% SPECIFIC 51Cr RELEASE
35
30
25
20
15
10
5
0
[CD2xCD2'xCD37]
[CD2xCD3xCD37]
[CD3xCD3xCD37]
[CD3xCD4xCD37]
[CD3xCD5xCD37]
1000
100
10
1
ANTIBODY (ng/ml)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
X	Cancer Detection and Prevention, Vol. 12, 1988, Ian G. Barr et al: "Retargeting of Cytolytic T Lymphocytes by Heteroaggregated (Bispecific) Antibodies ", see page 439 - page 450	1-3
Y		4-32
	--	
X	Chemical Abstracts, volume 107, no. 25, 21 December 1987, (Columbus, Ohio, US), Glennie, Martin J et al: "Preparation and performance of bispecific F (ab'y)2 antibody containing thioether-linked Fab'y fragments ", see page 597, abstract 234429b, & J. Immunol. 1987, 139(7), 2367-2375	1-3
Y		4-32
	--	
X	EP, A2, 0294703 (DANA-FARBER CANCER INSTITUTE, INC.) 14 December 1988, see page 4 - page 10	1-3
Y		4-32
	--	
X	EP, A2, 0241907 (THE GENERAL HOSPITAL CORPORATION) 21 October 1987, see the whole document	1-3
	--	
A	Proc.Natl.Acad.Sci., Vol. 86, May 1989, J M Rojo et al: "Physical association of CD4 and the T-cell receptor can be induced by anti-T-cell receptor antibodies ", see page 3311 - page 3315	4-32
	--	
X	Blood, Vol. 74, No. 2, 1989, Albertus W. Wognum et al: "An Enzyme-Linked Immunosorbent Assay for Erythropoietin Using Monoclonal Antibodies, Tetrameric Immune Complexes, and Substrate Amplification ", see page 622 - page 628	1-3
	--	

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/01335

SA 39700

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 01/11/90
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9001339	22/02/90	AU-D-	4062889	05/03/90
		EP-A-	0353960	07/02/90
		US-A-	4925648	15/05/90
EP-A2- 0336379	11/10/89	AU-D-	3242389	05/10/89
		JP-A-	1304356	07/12/89
WO-A1- 9004413	03/05/90	NONE		
WO-A1- 8911863	14/12/89	AU-D-	3752089	05/01/90
EP-A2- 0294703	14/12/88	JP-A-	64003128	06/01/89
EP-A2- 0241907	21/10/87	JP-T-	2500321	08/02/90
		WO-A-	87/06240	22/10/87
EP-A2- 0180171	07/05/86	JP-A-	61234779	20/10/86

For more details about this annex : see Official Journal of the European patent Office, No. 12/82

EPO FORM P0479

REVISED VERSION **

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :
C07K 15/28, G01N 33/563
A61K 39/395, C12P 21/08

A1

(11) International Publication Number: WO 91/03493

(43) International Publication Date: 21 March 1991 (21.03.9

(21) International Application Number: PCT/GB90/01335

(22) International Filing Date: 29 August 1990 (29.08.90)

(30) Priority data:
8919485.6 29 August 1989 (29.08.89) GB
9001949.8 29 January 1990 (29.01.90) GB

(71) Applicant *(for all designated States except US)*: THE UNIVERSITY OF SOUTHAMPTON [GB/GB]; Highfield, Southampton SO9 5NH (GB).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)* : GLENNIE, Martin, John [GB/GB]; The Tenovus Research Laboratory, Southampton General Hospital, Tremona Road, Southampton SO9 4XY (GB).

(74) Agents: WOODS, Geoffrey, Corlett et al.; J.A. Kemp & Co, 14 South Square, Grays Inn, London WC1R 5EU (GB).

(81) Designated States: AT, AT (European patent), AU, BB, BI (European patent), BF (OAPI patent), BG, BJ (OAP patent), BR, CA, CF (OAPI patent), CG (OAPI patent, CH, CH (European patent), CM (OAPI patent), DE* DE (European patent)*, DK, DK (European patent, ES, ES (European patent), FI, FR (European patent) GA (OAPI patent), GB, GB (European patent), HU, I (European patent), JP, KP, KR, LK, LU, LU (Europear patent), MC, MG, ML (OAPI patent), MR (OAPI pa tent), MW, NL, NL (European patent), NO, RO, SD SE, SE (European patent), SN (OAPI patent), SU, TD (OAPI patent), TG (OAPI patent), US.

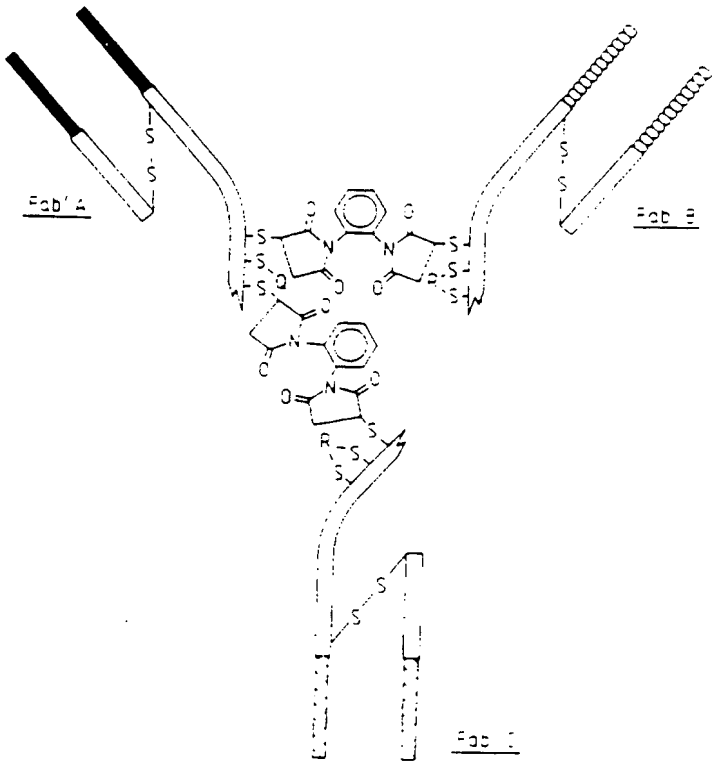
Published
With a revised version of the international search report.

(88) Date of publication of the revised version of th international search report:
19 March (19.03.92

(54) Title: BI-OR TRISPECIFIC $(FAB)_3$ OR $(FAB)_4$ CONJUGATES

(57) Abstract

Novel trimeric and tetrameric antibodies are disclosed, including bispecific and trispecific $F(ab)_3$ and $F(ab)_4$ antibodies. A simple and efficient method is described for the production of pure $F(ab'\gamma)_3$ antibodies, in which the individual antibody Fab' fragments are joined via stable thioether linkages. Hybrid molecules were constructed from mouse monoclonal antibodies with specificities for targetting cytotoxic effectors (human peripheral blood T cells) against ^{51}Cr-labelled chicken red blood cells. Fab' fragments from two of the chosen antibodies were first coupled via their hinge-region SH groups using o-phenylenedimaleimide (oPDM), this bispecific fragment was then linked, again via the hinge region using oPDM, to a third Fab' fragment.



TRISPECIFIC F(ab')3 ABC

REVISED VERSION

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 90/01335

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC5: C 07 K 15/28, G 01 N 33/563, A 61 K 39/395, C 12 P21/08

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
IPC5	A 61 K; C 07 K; G 01 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category *	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	WO, A1, 9001339 (IMMUNOMEDICS, INC.) 22 February 1990, see page 7 - page 14	1-11,15-32
P,X	EP, A2, 0336379 (ONCOGEN LTD) 11 October 1989, see the whole document	1-7,11,15
P,X	WO, A1, 9004413 (RESEARCH EXPLOITATION LIMITED) 3 May 1990, see page 22 - page 25	23-30
P,X	WO, A1, 8911863 (GLENNIE, MARTIN, JOHN) 14 December 1989, see the whole document	12-14

* Special categories of cited documents:[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance, the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance, the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
30th November 1990	14. 01. 92
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	Nicole De Bie

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Journal of Immunological Methods, vol. 120, 1989, Terry E. Thomas et al: "Specific binding and release of cells from beads using cleavable tetrameric antibody complexes ", see page 221 - page 231	1-3
Y	EP, A2, 0180171 (MASSACHUSETTS INSTITUTE OF TECHNOLOGY) 7 May 1986, see claims	1-3,5-7, 31,32

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO. PCT/GB 90/01335

SA 39700

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 30/04/91
The European Patent office is in no way liable for theseparticulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A1- 9001339	22/02/90	AU-D-	4062889	05/03/90
		EP-A-	0353960	07/02/90
		US-A-	4925648	15/05/90
EP-A2- 0336379	11/10/89	AU-D-	3242389	05/10/89
		JP-A-	1304356	07/12/89
WO-A1- 9004413	03/05/90	NONE		
WO-A1- 8911863	14/12/89	AU-D-	3752089	05/01/90
		EP-A-	0422043	17/04/91
EP-A2- 0294703	14/12/88	JP-A-	64003128	06/01/89
EP-A2- 0241907	21/10/87	JP-T-	2500321	08/02/90
		WO-A-	87/06240	22/10/87
EP-A2- 0180171	07/05/86	JP-A-	61234779	20/10/86